#39/JRW

Attorney's Docket No.: 06501-030001 / D1-003DP2-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Hiroaki Yamamoto
Serial No. : 09/305,390
Filed : May 5, 1999
Title : METHOD FOR PRODUCING OPTICALLY ACTIVE 4-HALO-3-HYDROXYBUTYRIC ACID ESTER

Art Unit : 1652
Examiner : R. Hutson

Commissioner for Patents
Washington, D.C. 20231

SECOND DECLARATION OF HIROAKI YAMAMOTO UNDER 37 C.F.R. § 1.132

1. I am the sole inventor of the above-identified U.S. patent application.

2. I am making this Declaration to provide relevant facts in support of the patentability of the subject matter claimed in the patent application.

3. I have read and understood the outstanding Office Action mailed March 12, 2002.

4. I understand that the Examiner contends that claims 7-10, 12, 14, and 23 are obvious over Matsuyama et al. in view of Peoples et al. (U.S. Patent No. 5,229,279) and Somerville et al. (WO 93/02187).

5. To demonstrate that other reductases do not all have the same substrate specificities, the following experiments were performed by me or at my direction:

Streptomyces violaceoruber IF0 15146 strain was cultivated in 50 mL of Bennett's Maltose medium (Yeast extract, 1 g/L; Beef extract, 1 g/L; NZ Amine, type A, 2 g/L; maltose, 10 g/L) at 30 °C, for 4 days. The cells were then collected by filtration and chromosomal DNA was prepared by the method described in Meth. Enzymol. 153:116-166, 1987.

Actinorhodin synthesis gene actIII (encoding keto acyl reductase (hereinafter "SvKR1"; DDBJ accession number, M19536)) was cloned using the primers SVR-ATG2 (AACCCATGGCCACGCAGGAC) and SVR-TAA2 (ATATCTAGATTAGTAGTTCCCCAGCCCGC) by PCR. The PCR was conducted by

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EV 044488750 US

April 7, 2003
Date of Deposit

subjecting 50 μL of a reaction solution (containing ExTaq buffer, 0.2 mM dNTP, 50 pmol of each primer, 2.5% DMSO, ExTaq 1U, and 125 ng chromosomal DNA), to 30 cycles of "denaturing" at 94 °C for 30 seconds, "annealing" at 55 °C for 30 seconds, and "elongation" at 72 °C for 1 minute.

The obtained PCR product was double digested using *NcoI* and *XbaI* and ligated with an *E.coli* expression vector pSE420D (Japanese Patent Application 2000-189170) that was double digested with the same restriction enzymes to construct the actIII gene expression plasmid pSE-SVR1. The cloned actIII gene was confirmed by sequencing and the sequence matched the sequence in the database.

E.coli strain HB101 was transformed with pSE-SVR1, and the obtained transformant was cultured in an LB medium (1% Bacto-Tryptone, 0.5% Bacto-Yeast extract, 1% NaCl, pH 7.2) containing 50 mg/L of ampicillin. When the turbidity at 600 nm was about 1.0, 0.1 mM of IPTG was added and further cultured at 30 °C for 4 hours.

The obtained cells were dispersed in a lysis solution containing 100 mM potassium phosphate buffer (pH 6.5), 0.02% 2-mercapto ethanol and 0.5 M NaCl, and the cells were disrupted by sonication. After centrifugation, the supernatant was collected as a cell-free extract.

A 1 mL reaction solution containing 100 mM potassium phosphate buffer (pH 6.5, 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) or reduced nicotinamide adenine dinucleotide (NADH), substrate, and cell-free extract was mixed and incubated at 25 °C. Absorbance at 340 nm was measured. A decrease in absorbance was indicative of a decrease in NADPH or NADH. Defining the IU as the amount of enzyme needed to catalyze a decrease of 1 μM of NADH or NADPH in 1 minute, 789 mU/mg-protein per 1 mL of medium was measured using 0.2 mM acetoacetyl-CoA as a substrate and NADH as the coenzyme.

The results shown in Table 1 below indicate that SvKR1 has a high specificity towards acetoacetyl-CoA, but hardly any activity towards ethyl-4-chloro acetoacetate that has a chloro-group at the 4th position (ethyl acetoacetate does not have this chloro-group).

TABLE 1: Substrate Specificity Of SvKR1

Coenzyme	Substrate	Substrate Concentration	Relative Activity
NADH	Acetoacetyl-CoA (AA-CoA)	0.2 mM	100%
NADH	Ethyl-4chloro acetoacetate (ECAA)	20 mM	0%
NADH	Ethyl acetoacetate (EAA)	20 mM	0.2%
NADPH	Acetoacetyl-CoA (AA-CoA)	0.2 mM	1.9%

Substrate specificity of α-ketoacyl-ACP reductase (BsKR1) derived from *Bacillus subtilis* was measured using *E.coli* HB101 cells transformed with the BsKR1 expression plasmid pSE-BSR1 and a cell-free extract prepared according to the method described in the specification and as described above, and measured as described in Example 3 of the specification using NADPH as the coenzyme (see, e.g., pages 21-26 of the specification).

When the activity of BsKR1 with the substrate ECAA was taken to be 100%, the activity with the substrate EAA (which does not have a chloro-group at the 4th position) was 4.8%, and the activity with the substrate acetoacetyl-CoA (AA-CoA) was 22%.

Substrate specificity of acetoacetyl-CoA reductase (ReAR1) derived from *Ralstonia eutropha* was measured using the *E.coli* HB101 cells transformed with the expression plasmid pSE-AER1 containing the ReAR1 gene and a cell-free extract prepared according to the method described in the instant specification and as described above, and measured as described in Example 3 of the specification using NADPH as the coenzyme (see, e.g., pages 21-26 of the specification).

When the activity of ReAR1 with the substrate ECAA was taken to be 100%, the activity with the substrate EAA (which does not have a chloro-group at the 4th position) was 1.3%, and the activity with the substrate acetoacetyl-CoA (AA-CoA) was 39.3%.

6. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of any patent issuing from the present patent application.

March 25, 2003
Date

Hiroaki Yamamoto
Hiroaki Yamamoto